21.027

Construction and Histopathological Characterization of Multiple Virulent Genes Mutant of *V. cholerae*: To Understand the Enteropathogenesis of Cholera

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Cholera is caused by toxigenic *V. cholerae* that secretes the cholera toxin (CT) encoded by *ctx* gene. Although CT is the major toxin, accessory toxins *rtx* gene encoding RTX toxin and *hap* gene that secretes hemagglutinin/protease (HA/P) have been shown to elicit mild diarrhoea and inflammatory reactions. The present study was designed to produce a *V. cholerae* O139 vaccine candidate having multiple virulent genes mutation and study their enteropathogenesis in animal model. The *ctx*, *rtx* and *hap* genes were mutated individually by allele replacement method and the vaccine candidate were named as VCUSM14P, VCUSM10P and VCUSM17P respectively. A multitoxin - deficient mutant (VCUSM22P) was created that have all 3 genes mutated. The mice colonization ability in all the above mentioned individual and multitoxin - deficient mutants were good and were 1 log lower that of wild type (WT) strain. In ileal loop assay, no fluid accumulation were seen in VCUSM14P and VCUSM22P which have the *ctx* gene mutated, while VCUSM10P and VCUSM17P showed more fluid accumulation as seen in the WT strain due to the presence of intact *ctx* gene. The histopathological studies, correlated with the fluid accumulation data wherein the tissue sections infected with VCUSM14P and VCUSM22P showed the presence of intact villi, intestinal gland with no damage in the submucosa, muscularis or serosal layer. But VCUSM10P and VCUSM17P caused sloughing of the villi, mild to moderate haemorrhage, congested blood vessel in submucosa and PMN in the lamina propria, as in tissue infected with WT strain. The histopathological features were further confirmed by immunohistochemical analysis. Thus it is clear that the mutation of only accessory toxins (RTX and HA/protease) caused only modest changes in virulence. While multitoxin - deficient mutant (VCUSM22P) are potent vaccine candidate which shows better colonization, low virulence with no enteropathogenic, cytopathic and haemolytic effects.

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In-vitro and *In-vivo* Studies on the Effect of Using Single and Combinations of Antibiotics on Different Phases of Bacterial Growth

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Background: In our few working Sudanese microbiological laboratories, sensitivity testing for isolated organisms is usually performed using the disc method. The disc method tests for the sensitivity of a single antibiotic at different concentrations. Accordingly the antibiotics are judged whether they are effective (sensitive) or ineffective (resistant). In this test, the bacteria used are always in the stationary phase or declining phase. This situation does not answer several important questions.

Is it possible to overcome bacterial resistance by using different combinations of commonly used antibiotics? Do bacteria at different phases of growth, starting from lag phase to declining or death phase behave similarly in their sensitivity to various combinations of antibiotics? Are the effects of these combinations the same *in vivo* as *in vitro*?

The aim of the present study is to render available a clue about the use of combinations antibiotics so as to find out the most effective time of starting the treatment by antibiotics, at the beginning of onset of disease or latter when symptoms have clearly developed in both man and animals. This aim can be achieved through the following objectives:

1. To test the sensitivity of selected resistant Gram negative organisms to combinations of commonly used antibiotics in Sudan.
2. To compare the sensitivity of bacteria at different phases of growth to antibiotics when using single antibiotic or combinations of antibiotics.
3. To monitor *in vivo* the sensitivity of selected bacteria at different phases of growth to single and combinations of antibiotics, which were found to be effective *in vitro*.

Methods: Bacterial strains and susceptibility testing

Pasturella multocida local sensitive strain to most of the antibiotics which are commonly used in Sudan had been used in these studies.

Antibiotics Erythromycin and tetracycline each separately and in combination were used in these experiments.

In vitro studies (growth curve with antibiotics)

Ten-ml sterile bottles of nutrient broth were inoculated by a colony or part of a colony of *Pasteurella multocida*. The inoculum was emulsified carefully and the bottle was incubated at 37 °C for 18 hours. Eight bottles containing 99 ml Mueller Hinton Broth each were prepared and were inoculated with one ml from the overnight culture. The eight were labeled time zero, one hour, two hours, four hours, six hours, eight hours and twenty four hours. From the first bottle (at zero time) one ml was discarded and replaced by one ml normal saline containing the tested antibiotic of 1 MIC concentration (erythromycin = 2 μgm/ml, tetracycline = 4 μgm/ml and combination of erythromycin/tetracycline = 0.125/0.25 μgm/ml). The antibiotic was allowed to act for 15 minutes then the viable count was done. The other seven bottles were incubated at 37 °C for 1 hour, 2 hours, 6 hours, 8 hours and 24 hours respectively. After the inoculum time 1 ml was discarded and replaced by 1 ml normal saline containing the antibiotic of 1 MIC concentration. Then after 15 minutes viable count using Miles and Misra (1938) method was done.

The results of the viable count were compared with a standard curve for *Pasteurella multocida* (control) without the addition of any antibiotic.

In vivo studies.

Animals: White mice weighing approximately 23—28 g were used throughout this study. Animals were allowed, at least 72 h after delivery, to acclimatize to laboratory surroundings before experimentation.

Minimum lethal dose determination (MLD).

The minimum lethal dose, the lowest diluton of organisms at which 100% mortality occurred, was determined by intraperitoneal injection of groups of mice (three per group) with 0.5 ml of bacteria in Mueller-Hinton broth at serial 10-fold dilutions.

Animals were observed for 24 hours, and the rate and number of deaths at each dilution were recorded.

For each MLD determination, a control group of mice received normal saline instead of the bacterial suspension.

Experimental infection: Infection was produced in by intraperitoneal injection of 0.1 ml of broth containing approximately 1.2×10^7 CFU of bacteria per ml ($10 \times$ MLD).

Antibiotics treatment: The mice were divided at random into three groups; each group contained three sub groups each consisting of three animals.

In the first group of experiments (group 1), 0.1 ml of tetracycline was administered intravenously at 0 h (= time of infection) and three hours afterwards at the indicated dose of 25 mg/kg of body weight to the first sub group.

The second sub group set of experiments 0.1 ml of erythromycin was administered intravenously at 0 h (= time of infection) and three hours afterwards at the indicated dose of 30 mg/kg of body weight to the second sub group.

The last sub group was performed with erythromycin and tetracycline in combination.

For each sub group, a control group of mice received normal saline instead of the bacterial suspension.

Animals were observed for 24 hours, and the rate and number of deaths at each dilution were recorded.

Results: In vitro studies on effect of using single and combination antibiotics on different bacterial growth phases.

The results shows that the maximum numbers of viable cells of *Pasteurella multocida* were killed when erythromycin was added to 6 hours growing cells. Addition of tetracycline resulted in decrease in the viable cell count at 1-2 hours and 6 hours. When combination of two antibiotics with lower concentration than each singly was used, the maximum numbers of bacteria were killed at 2 hours and 6 hours.

In vivo studies on effect of using one or two antibiotics on different bacterial growth phases.

The results show that 100% of the animals survived when combination of erythromycin and tetracycline was used either half an hour after infection or after 8 hours. Using the combination after 3 hours of infection resulted in the cure of 67% of the infected animals.

When using erythromycin alone, only 33% of the infected animals survived when the dose was administrated after 3 hours of infection. Where as when using tetracycline alone, 33% of the animals survived when the dose is administrated at the beginning of infection.

Conclusion: From the above results we can conclude that *Pasteurella multocida* is sensitive to combination of erythromycin and tetracycline *in vivo* as well as *in vitro*. We can also conclude that different antibiotics have different effects on the cells of *Pasteurella multocida* at different phases of growth.

The experiments reported here also suggest that the variable of bacterial growth phase should be further evaluated and standardized for quantitative testing of antibiotic susceptibility *in vitro*. Additional information is needed concerning the importance of the growth phase in killing by agents other than what we used either singly or in combination and on the correlations between the responses of bacteria to antimicrobial agents *in vitro* and *in vivo*.

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Obstetrical-Gynecological, Surgical and Sexually Transmitted Infections (Poster Presentation)

22.001

Prevalence of *Chlamydia trachomatis* Antibodies Among Infertile Subjects in Anambra State, Nigeria

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Background: Sexually transmitted diseases (STDs) remain major public health problem in Nigeria, as in other parts of the world. Urogenital Chlamydial infections, caused by Chlamydia trachomatis, are considered the most prevalent non-gonococcal STD. Most urogenital chlamydial infections are asymptomatic and failure to diagnose and treat the infection at an early stage may result in serious complications and sequalae, such as infertility. Information regarding the involvement of urogenital chlamydial infections in infertility is lacking in Anambra State of Nigeria, and hence the need for this study.

Materials and Methods: The study population comprised males (42) and females (100), who were receiving treatment for infertility between August and December, 2003; ninety of the subjects were cases of primary infertility, while 52 had secondary infertility. Twenty three (61%) were asymptomatic. Questionnaires were served, and sera appropriately collected. Chlamydia antibodies were sought by ELISA techniques, using Chlamydia trachomatis IgG serodiagnostic test kits, as well as IgM kits.

Results: Thirty-eight (27%) of the patients were seropositive for C. trachomatis antibodies. Chlamydia antibodies were slightly more prevalent in infertile females (28 or 28%) than males (10 or 24%). Majority (33%) of seropositive individuals had 2 or more sexual partners. Rate of seropositivity was highest among individuals in 26-40yrs age bracket (28 or 32%). Presence of Chlamydia antibodies was most prevalent in males and females employed in corporate organizations (44%), followed by those in private business (30%). Seropositivity was more frequently observed among those with secondary infertility than in those with primary infertility. Prevalence of antichlamydial antibodies reduced with increasing exposure to secular education.

Conclusion: It could be concluded that *Chlamydia trachomatis* - associated infertility is a problem in Anambra state of Nigeria, particularly among those having multiple sex partners. It is suggested that Chlamydia tests be included among the routine medical investigations in this locality.

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